

Letter: Discoveries

Molecular data support an early shift to an intermediate-light niche in the evolution of mammals

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Abstract

The visual ability and associated photic niche of early mammals is debated. The theory that ancestral mammals were nocturnal is supported by diverse adaptations. However, others argue that photopigment repertoires of early mammals are more consistent with a crepuscular niche, and support for this also comes from inferred spectral tuning of middle/long wavelength-sensitive (M/LWS) opsin sequences. Functional studies have suggested that the M/LWS pigment in the ancestor of Mammalia was either red- or green-sensitive; however, these were based on outdated phylogenies with key lineages omitted. By performing the most detailed study to date of middle/long-wave mammalian colour vision, we provide the first experimental evidence that the M/LWS pigment of amniotes underwent a 9nm spectral shift towards shorter wavelengths in the Mammalia ancestor, exceeding predictions from known critical sites. Our results suggest early mammals were yellow-sensitive, possibly representing an adaptive trade-off for both crepuscular (twilight) and nocturnal (moonlight) niches.

Key words: red/green visual pigment, spectral sensitivity, adaptation, temporal niche, mammals

Running head: Intermediate-light adaptation for early mammals

The spectral sensitivity of early mammals is controversial, but can shed light on evolutionary shifts in ecological niche. The long-held theory that ancestral mammals occupied a nocturnal niche to avoid diurnal archosaurs ('nocturnal bottleneck') is supported from analyses of physiological and behavioural adaptations as well as eye morphology (Heesy and Hall 2010; Hall et al. 2012; Gerkema et al. 2013). Recent ancestral reconstructions of activity patterns in mammals have also suggested an early nocturnal period, leading to diurnality in the Cenozoic (Maor et al. 2017). However, others have argued that the photopigment repertoires of early mammals are more consistent with activity at mesopic conditions at dusk and dawn, rather than a nocturnal environment (Davies et al. 2012a), and this idea has also received support from analyses of molecular evolution (Wu et al. 2017).

Proposed evidence for nocturnality in early mammals also comes from studies of opsin genes, which encode the photopigment proteins of rod and cone cells in the retina (Bickelmann et al. 2015; Emerling et al. 2015). Vertebrates typically possess rods that contain a rhodopsin (RH1) pigment sensitive to dim-light, and four types of cones each containing a different opsin, conferring colour vision. These opsins vary in spectral sensitivity; the middle or long wavelength-sensitive (M/LWS or red/green) pigment, a rhodopsin-like (RH2 or green) pigment, and short wavelength-sensitive type 1 (SWS1 or ultraviolet/violet) and type 2 (SWS2 or blue) pigments (Davies et al. 2012a). Comparative analyses show that the RH1 pigment was retained during the origin of mammals, and are consistent with suggestions that this pigment likely relates to greater activity in dim-light conditions (Bickelmann et al. 2015). In contrast, analyses of opsin gene sequences reveal that RH2 was lost in early mammals, followed by SWS1 and SWS2 in early monotremes and therians, respectively (Davies et al. 2012a). The functional M/LWS pigment has been retained in monotremes, marsupials and placentals (Davies et al. 2012a), with only a few exceptions found in animals that inhabit extreme scotopic environments (Meredith et al. 2013; Emerling and Springer 2015), implying a critical function in visual perception.

The spectral tuning of M/LWS pigments can be broadly estimated from five critical amino-acid sites (180, 197, 277, 285 and 308) that are known to account for the wavelength of maximum absorption, λ_{\max} (Yokoyama and Radlwimmer 2001; Yokoyama et al. 2008b), although this property might also be influenced by other sites (Asenjo et al. 1994) as well as by the presence of chloride ions (Davies et al. 2012b). Applying the so-called 'five-sites' rule, the M/LWS pigment in extant lineages has a λ_{\max} ranging from ~510 (green) to ~560nm (red) (Yokoyama et al. 2008b). On the other hand, the spectral sensitivity of the primitive forms of this pigment in early mammals is debated, and thus the visual ecology of these lineages is also open to question. Experimental work on ancestral opsin sequences suggested that the M/LWS pigment of early vertebrates was tuned to red light ($\lambda_{\max} = \sim 560\text{nm}$) (Yokoyama and Radlwimmer 2001; Yokoyama et al. 2008b), while that of the ancestral mammal was either red- (~560nm) or green-sensitive (~530) (Yokoyama and Radlwimmer 1999; Yokoyama and Radlwimmer 2001; Yokoyama et al. 2008b). However, the phylogenies from which these values were based are now known to be incorrect, and also did not contain sequences from prototherians.

Newly available whole genome sequence data, together with an improved understanding of mammalian phylogenetic relationships (Springer et al. 2003; Miller-Butterworth et al. 2007; McGowen et al. 2009; Pyron 2010; Fabre et al. 2012; Hassanin et al. 2012; Nyakatura and Bininda-Emonds 2012; Mitchell et al. 2014; Pozzi et al. 2014), provide opportunities to reconstruct ancestral gene sequences with greater reliability and confidence than ever before (see supplementary Materials and Methods). Here we obtained and analysed *M/LWS*

sequences from 80 mammal species spanning 21 orders, and performed reconstructions of the ancestral opsin proteins to infer spectral sensitivities of five primitive forms of the protein. These forms corresponded to the ancestors of the each of major lineages of mammals: the placental mammals (subclass Eutheria), marsupials (subclass Metatheria) and monotremes (subclass Prototheria). Additionally we examined the common ancestors of all therians (Eutheria+Metatheria), of all mammals (Mammalia), and finally, of all mammals, birds and reptiles (Amniota) (supplementary fig. S1).

Our ancestral sequence reconstructions of M/LWS indicated that the five critical sites in the ancestor of amniotes was SHYTA (respective residue positions 180, 197, 277, 285 and 308), in agreement with previous inferences based on sequence data (Yokoyama and Radlwimmer 2001). For each of the three main sub-class lineages of mammals, as well as for the Mammalia, the five critical sites were inferred to be AHYTA (fig. 1). This latter result agrees with a recently reported sequence based on much fewer extant species (Wu et al. 2017). Previously, three different ancestral forms for Mammalia have been proposed, with varying λ_{\max} values estimated from *in vitro* assays: SYYTA (531 or 536nm) (Yokoyama and Radlwimmer 1999; Yokoyama et al. 2008b), AYYTA (533nm) (Yokoyama and Radlwimmer 2001) and SHYTA (~560nm) (Yokoyama and Radlwimmer 2001; Yokoyama et al. 2008b), with the major shift caused by the loss of a chloride binding site (H197Y) (Sun et al. 1997). In contrast, λ_{\max} values for M/LWS photopigments with SHYTA and AHYTA critical sites have been estimated as 557-565nm and 550-558nm, respectively (Yokoyama et al. 2008b).

Our finding implies that a spectral shift to middle wavelengths in the M/LWS pigment of early mammals almost certainly occurred before the split between Prototheria and Theria, and that this shift involved a change from SHYTA to AHYTA. The S180A substitution at the base of mammals was well-supported under amino-acid and codon substitution models, based on a constrained species topology (Bayesian posterior probability [BPP] = 0.87 and 0.93, respectively) as well as based on *de novo* Bayesian and maximum likelihood (ML) tree reconstructions (BPPs = 0.86 and 0.99, respectively, obtained for both topologies; supplementary fig. S2). Incomplete support reflects the presence of different residues at the first critical site in the echidna and platypus (serine and alanine, respectively; Supplementary fig. S3). Additional indirect support for our proposal that the amino-acid residue in the ancestor of Mammalia is alanine, rather than the serine seen in the echidna, comes from phylogenetic reconstructions that show the echidna likely evolved from a platypus-like ancestor (Phillips et al. 2009).

Predicted spectral tuning from critical sites can occasionally deviate from actual absorption values (Yokoyama et al. 2008b). Therefore, to verify our results we expressed the ancient opsin genes *in vitro* and then measuring the sensitivities of the resurrected pigments. Functional assays of ancestral M/LWS pigments revealed λ_{\max} values of 551nm for Mammalia, and 551 to 553nm for therians, eutherians, metatherians and prototherians (fig. 1 and supplementary fig. S4). Thus all of these ancient forms were yellow-sensitive (550-556nm) (Jacobs 1996; Cropp et al. 2002). Using predictions based on the ‘five-sites’ rule (Yokoyama et al. 2008b), we also estimated λ_{\max} values of pigments for the living species in our dataset, and found 39 out of 78 dichromatic species were sensitive to yellow light, 33 to green, and six to red (total range <509 to 564nm; see supplementary fig. S3). These changes are likely to have arisen due to specific selection pressures that in most cases are poorly known; thus *in vitro* assays for the pigments in the future will be essential for refining our understanding of these sensory and ecological adaptations.

It is important to note that ancestral reconstructions based on ML method are probabilistic and may under-represent the presence of rare variant residues (Pollock and Chang 2007). Although we cannot rule out slight variation between our inferred primitive sequences and those of the true ancient genes, our inferred ancestral reconstructions of mammalian ancestor were robust to two models of amino-acid substitution. Given the fact that the observed variation (3-56 amino-acid differences) among the five primitive pigments accounted for minor shifts in λ_{\max} ($\leq 2\text{nm}$), it is likely that any additional substitutions that occurred between the ancestors of all mammals and those of the sub-class lineages will have had negligible functional impacts. Indeed, we repeated our *in vitro* assays for an alternative ancestral Mammalia M/LWS pigment that was recovered using a codon model, and found that two amino-acid differences (I104V and I153V) also only led to minimal changes in sensitivity (supplementary fig. S4).

Our finding that ancestral forms of M/LWS in Mammalia and Amniota differ by 9nm in their maximum absorption (based on λ_{\max} of Mammalia versus Amniota) is interesting given that changes at the first critical site are normally associated with spectral changes of only $\sim 5\text{nm}$ (Yokoyama et al. 2008b). To verify this experimentally, we modified the ancestral amniote sequence by introducing a single S180A substitution, and found that the associated λ_{\max} -shift was just 5nm. We then repeated this experiment by introducing all of the other 25 derived amino acids seen in the Mammalia ancestor (all of which occur outside of the five critical positions) while retaining the ancestral serine at position 180. This assay led to a 4nm shift, and thus we can conclude that one or more of these 25 residue differences between the two ancestral forms have contributed to an additional λ_{\max} -shift of around 4nm (supplementary fig. S4). Previous work has shown that substitutions other than at the five critical sites also contribute to spectral shifts between human red and green pigments (Asenjo et al. 1994); thus more work is needed to determine the hidden critical site(s) in the M/LWS pigment, and to assess whether they act singly or in combination with each other.

A 9nm λ_{\max} -shift in M/LWS exceeds the previously reported adaptive λ_{\max} -shift of 4-5nm in the vertebrate RH1 pigment (Yokoyama et al. 2008a). Moreover, in humans, individuals who carry the variant M/LWS allele containing AHYTA (552nm) perceive colour differently from those possessing the more common allele (SHYTA; 557nm) (Merbs and Nathans 1992; Winderickx et al. 1992), and this phenomenon includes red-green colour blind individuals who are functionally equivalent to dichromatic mammal species (Sanocki et al. 1993). We therefore suggest that observed red to yellow shift in early mammals will have had important biological consequences, and that the subsequent retention of this yellow-sensitivity across ~ 150 million years implies functional conservation in the early radiation of mammals (fig. 1). This directional shift is consistent with adaptation to twilight conditions, when the light is enriched with blue-green wavelengths (Davies et al. 2012a; Wu et al. 2017). On the other hand, previous findings suggest that, at this evolutionary stage, spectral shifts were unlikely to have occurred in either SWS1 (Yokoyama et al. 2016) or SWS2 (Yokoyama and Tada 2003; Davies et al. 2007; Wakefield et al. 2008) pigments (fig. 1). Interestingly, while ancestral reconstructions performed for the RH1 pigment of early mammals also revealed no spectral shift at this time, functional assays suggested a change in rate of retinal release for this pigment, which might also have arisen as an adaptation to dim-light conditions (Bickelmann et al. 2015). In general, the possibility that visual pigments have undergone molecular evolution for other non-spectral roles cannot be ruled out (Castiglione et al. 2017; Dungan and Chang 2017).

Our results are consistent with the recent suggestion that the inferred complement of opsins in

early mammals, and their associated retention of colour vision, is not fully consistent with nocturnal bottleneck theory, but instead is better explained by selection for a mesopic/twilight niche (Davies et al. 2012a). If this theory is correct, however, then it raises the question of why the magnitude in the spectral shift of early mammals from 560 to 551nm was not greater, especially given that twilight can be enriched with wavelength of 450nm (Johnsen et al. 2006; Melin et al. 2012). Indeed, the M/LWS pigment of some muroid rodents and the rabbit, both of which lost the chloride binding site by H197Y substitution, are tuned to ~510nm (Sun et al. 1997; Radlwimmer and Yokoyama 1998), and are thus more sensitive to blue-green rather than yellow light. We hypothesize that an alternative explanation is that the spectral shifts in early mammals represent a trade-off to exploit the photic conditions of both twilight at dusk and dawn, and moonlight at night, enriched with wavelengths of 450nm (Johnsen et al. 2006; Melin et al. 2012) and 560nm or longer (Melin et al. 2012; Veilleux and Cummings 2012), respectively. In this regard, it is noteworthy that some extant mammals are known to use both twilight and moonlight, such as the aye-aye (Melin et al. 2012) and the woolly lemurs (Veilleux et al. 2014). Thus we conclude that molecular evolution of the M/LWS pigment was pivotal to the adaptation of mammalian colour vision to the intermediate-light conditions of crepuscular (twilight) and nocturnal (moonlight) environments.

Figure legend

Fig. 1. The evolution of visual pigments during the origin of mammals. On each of the six focal ancestral nodes, the five critical sites for the M/LWS pigment are shown, together with the corresponding dark-light difference spectra obtained by functional assays. Additionally, amino-acid substitutions (transmembrane domains I to VII) are shown along the ancestral branches, with the verified critical substitution S180A indicated underlined. Red and yellow circles represent long wavelength and middle wavelength-shifted sensitive pigments, respectively. The evolution of the five visual pigment groups M/LWS, RH1, RH2, SWS2 and SWS1 are shown in the inset, with truncated branches representing inactivated visual pigments.

Supplementary Material

Supplementary Materials and Methods, tables S1 and S2, figures S1-S4 and data used for ancestral sequence reconstruction are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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